ELECTROSPINNING, PHOTOCROSSLINKING OF ARYLAZIDE CHITOSAN NANOFIBERS AND APPLICATION AS BIOCATALYST SUPPORT MATRIX

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ABSTRACT

A process yielding water stable chitosan nanofiber mats is described and their significance in the field of biocatalyst immobilization is proven. Electrospinning was adapted to produce nanofibers from chitosan derivatives bearing photoreactive arylazide groups. These photoreactive groups of chitosan were then crosslinked by irradiation with UV-light. This stabilizes the fiber morphology even upon incubation in water and provides an alternative to crosslinking with harmful chemicals. Applicability of the fiber mats as enzyme immobilization matrix was demonstrated, using β-*D*-galactosidase as model enzyme. Although an initial leaching of enzyme was observed, after 40 days of incubation a significant portion of active enzyme is still present on the fibers. Thus, the fiber mats are suitable matrices to support enzymes and can revolutionize future enzymatic production processes of e.g. active pharmaceutical ingredients.

Keywords: arylazide chitosan, electrospun nanofibers, photochemical crosslinking, enzyme immobilization.

INTRODUCTION

To this day, applications of enzymes in the production of active pharmaceutical ingredients (API) are limited by losses in enzyme activity or difficulties in reusability and handling. Enzyme immobilization onto a structural and porous support may help to overcome these obstacles (Sheldon, 2007). Chitosan nanofibers are promising candidates as immobilization matrices for biocatalysts because of their high surface-to-volume ratio in combination with an ease of handling. This may lead to the development of a new class of biocatalyst-supports (Misson et al., 2015). As chitosan is still one of the most promising polysaccharides derived from nature, its electrospinning process has been studied intensively for the last two decades (Ding et al., 2014). An important improvement came from (Zhang et al., 2008) by blending of chitosan with poly(ethylene oxide) (PEO) of a very high molecular weight. This enabled production of nanofibers. However, most of the previously spun nanofibers tend to lose structure completely upon contact with water. Chemical modification of chitosan with photo-reactive crosslinking groups before electrospinning can help to solve this problem (Hadler et al., 2017). In this way an UV-light induced crosslinking is possible and a very good alternative to common crosslinking agents such as Genipin or Glutaraldehyde. The crosslinked hydrophilic, but water stable nanofibers could be used for immobilization of enzymatic catalysts to improve production processes for various API's.

RESEARCH CONCEPT

All chemicals were obtained from Sigma Aldrich unless otherwise stated. Poly(ethylene oxide) (PEO, M_v = 5.000 kDa) was used as obtained. Chitosan (CsH, M_w = 190 – 310 kDa, 15 – 25 % degree of acetylation) was purified, using a method of (Gan and Wang, 2007). Arylazide chitosan (CsH-Az) was synthesized by 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholi nium chloride (DMT-MM) mediated aqueous amide coupling of sodium-4-azidobenzoate and amine functions of chitosan by a method of (Hadler et al., 2017). Spinning solutions were prepared using blends of CsH-Az and PEO in a solvent system consisting of 3 % acetic acid in deionized water and methanol (10:1 v/v) as a co-solvent. The blends had a PEO content of 5 % (w/w) and were prepared with an overall polymer concentration of 4 % (w/v). All fibers were spun on a custom-made electrospinning device (see Figure 1) by extruding spinning solution through a flat cannula (I, B/BRAUN, inner diameter = 0.2 mm) to a plastic syringe (II, B/BRAUN, 5 mL). A syringe pump (III, HLL LANDGRAF LABORSYSTEME, *LA 30*) was used to maintain a constant flow rate. A fiber-forming jet was achieved by applying a high electric potential (IV, HEINZIGER, *LNC-30000-2 pos* high voltage generator) between the spinneret and the collector. All fibers were collected on a rotating spherical alumina cylinder (V, IKA LABORTECHNIK, *RW20 DZM* stirrer, 1000 rpm) with a height of 5 cm and a radius of 3 cm. The collector was covered in a strip of plastic mesh

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(WINDHAGER, *Fiberglas Anthrazit* (fly-screen) which improved the handling of the fibers. The distance between spinneret and collector, the electrical potential and the flow rate of spinning solution were varied in order to achieve a stable production process. Produced fibers were analyzed by IR-spectroscopy and SEM images.

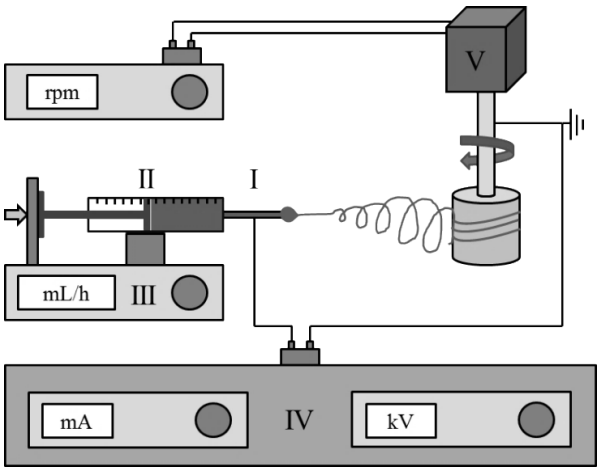

Figure 1: Schematic setup of custom-made electrospinning device (I = cannula, II = plastic syringe, III = syringe pump, IV = high voltage generator, V = collector).

Samples of the fiber meshes were photo-crosslinked by subjecting them to light of a mercury vapor UV-lamp (ORIEL, 100 mW/cm^2) for a given time. The lamp was equipped with a water IR-filter and a glass UV-filter (ν_{max} = 330 ± 70 nm). Completion of crosslinking was evaluated by recording IR-spectra of samples. Water stability tests were carried out by incubation of samples in deionized water for a given time and subsequent drying with a N_2-stream. Scanning electron microscopy (SEM) images were used to evaluate water stability of fibers. Samples with 100 mm^2 of photocrosslinked fiber mesh were used for immobilization of β-*D*-galactosidase (β-Gal) from *A. oryzae* as model enzyme via ionic interaction. Three samples per experiment were washed with citrate-phosphate-buffer (CP) at pH = 4.5 for 24 h, subsequently incubated with 0.506 units of β-*D*-galactosidase in CP at r.t. for 24 h and stored in Millipore water in fridge until further use. Remaining activity of immobilized enzymes was determined via O-Nitrophenyl-β-D-galactopyranoside (ONPG) at 0, 20 and 40 d. This was done by incubation of samples in ONPG (5 mM, 1 mL, 30 °C, 700 rpm inversion) and subsequent stopping of reaction after 10 min with borate buffer. UV-VIS-spectroscopy at 420 nm was used to determine the amount of cleaved ONPG and calculate the remaining activity of immobilized β-Gal relative to initial activity in immobilization solution before contact with samples.

RESULTS AND DISCUSSION

By reproducing electrospinning experiments from (Zhang et al., 2008) with our custom-made electrospining setup, we could obtain chitosan (CsH) nanofibers, that showed poor stability in water incubation tests. A complete loss of nanofiber morphology was observed after contact with water. This phenomenon has been described before and highlights the need of a crosslinking method to increase the water stability of the fibers. Previous approaches consist of a treatment of fibers with a crosslinking agent such as Genipin (Li et al., 2015), Glutaraldehyde (Schiffman and Schauer, 2007) or Tripolyphosphate (Sarkar et al., 2013). All of these methods have the drawback that fibers have to be treated with a chemical and can potentially be damaged during this process. A more elegant method is crosslinking of chitosan fibers by irradiation with UV-light. In order to enhance CsH with photocrosslinking functionalities, arylazide groups were introduced by a DMT-MM mediated aqueous amide coupling of sodium 4-azidobenzoate and amine functions of CsH. The resulting structure of chitosan-azide (CsH-Az) is shown in Figure 2. For this study we produced CsH-Az with a degree of modification (DM) in a range of 1 – 10 %. DM is defined as the fraction of modified repetition units over the sum of all repetition units in CsH (see Figure 2).

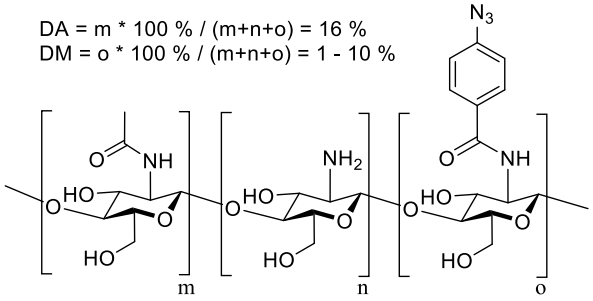

Figure 2: Schematic structure of CsH-Az: (m = acetylated glucosamine, n = glucosamine, o = arylazide modified repetition unit).

Modification of glucosamine repetition units with arylazide groups changes the properties of chitosan in a spinning solution drastically. Therefore, established parameters of the spinning process have to be adapted. Significant improvements of the spinning process and resulting fibers were achieved by substitution of DMSO

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with Methanol (MeOH) as a co-solvent. This led to a decrease of viscosity of the spinning solution and by this, to a much more stable spinning process. We were able spin solutions of CsH-Az/PEO-blends (w/w = 95/5, DM = 0 % / 1.9 % / 3.8 % / 9.0 %) using the improved AcOH/MeOH-solvent system in a stable process (spinning time over 3 h, rotating drum collector at 1000 rpm, voltage of 14 kV, cannula-collector-distance of 25 cm, solution flow rate of 1 mL/h). Best results were achieved for the CsH-Az-6/PEO-blend (DM of 3.8 %). The resulting fibers, in contrast to other CsH-Az/PEO-blends, had nearly no bead-like defects and were well oriented in a mostly parallel and straight manner. The average diameter was 640 +/- 290 nm. A SEM image of this morphology is shown in Figure 4. Fibers of CsH-Az/PEO-blends with a DM of 1.9 % or 9.0 % respectively had bead-like defects and the complementary spinning process was less stable than for a DM of 3.8 % (CsH-Az-6). This clearly indicates that a change in DM of CsH-Az requires an adaption of the specific process conditions as it changes the behavior of the polymer chains in the spinning solution. After spinning, CsH-Az/PEO-fibers can be crosslinked via irradiation with UV-light. This was done with a Hg-vapor UV-lamp at a light intensity of 100 mW/cm^2. We were able to crosslink samples within a time spectrum of 30 – 180 s as established by ATR-IR-spectra, SEM-images and water stability tests.

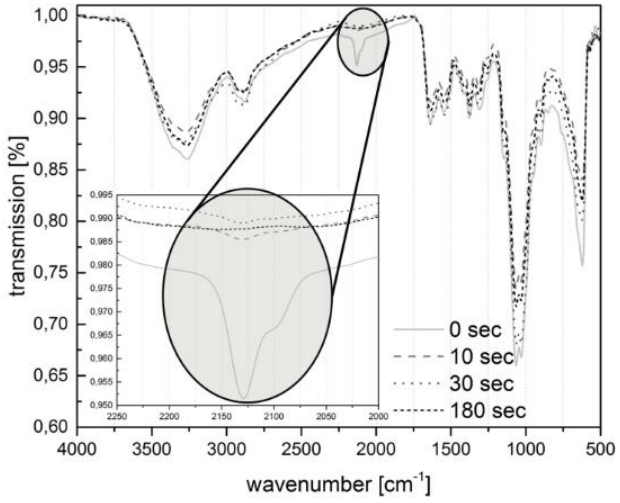

Figure 3: Decreasing intensity of azide signal (magnified section) in ATR-IR-spectra of CsH-Az/PEO fiber meshes at varying UV-light exposure times during photo-crosslinking process.

ATR-IR-spectra of fiber samples before and after irradiation show decrease of a signal at 2130 cm^{-1} (see Figure 3). This signal corresponds to azide groups of CsH-Az. Further evidence for a successful crosslinking process was provided by incubation experiments in water. CsH as well as CsH-Az is hydrophilic and can be dissolved in slightly acidic water conditions (pH 4-5). However, crosslinked chitosan forms a hydrogel in water. The crosslinked CsH-Az/PEO fibers behave similarly: Samples examined at macroscopic level during and after incubation showed complete water stability for over 24 h. The dry white fiber mesh becomes semi-transparent during incubation and reverses back during drying. Examination of the incubation process by light microscopy reveals a significant increase in fiber diameter during incubation. SEM-images of the fiber meshes after subsequent drying showed partial fusing of fibers but retention of general fiber mesh structure. A typical morphology of such partially fused fibers is shown in Figure 4.

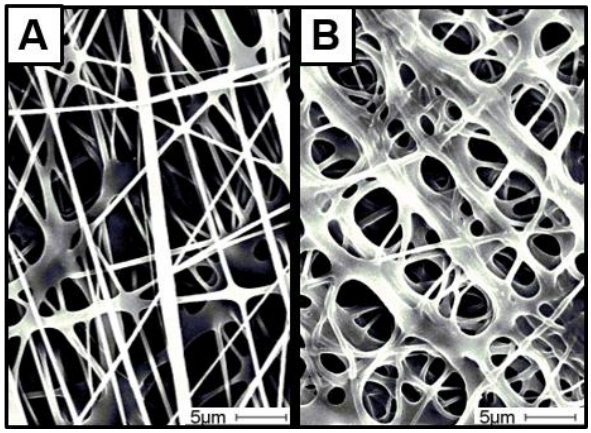

Figure 4: SEM image of fibers from CsH-Az-6/PEO-blend spun from AcOH/MeOH solvent system before (A) and after (B) crosslinking and incubation in deionized water.

Incubated fiber samples (Figure 4) appear, compared to non-incubated fibers, washed-out and fused together. A lot of pores have formed in between fusing fibers. Some fibers seem to stick together and form fiber bundles. This morphology did not change with irradiation time or incubation time. It seems that morphology changes happen during first contact with an incubation solution. To demonstrate a potential application of CsH-Az nanofibers, β-Gal was immobilized via ionic interaction and adsorption on fiber surfaces as proof of concept. As analysis of exact enzyme loading on fibers is not trivial, relative activity standardized with activity of free enzyme in CP buffer at pH = 4.5 was used. Initially directly after incubation, we found a high relative activity of 75 % in case of CsH-Az-6 and 80 % for CsH-Az-9 (Figure 5).

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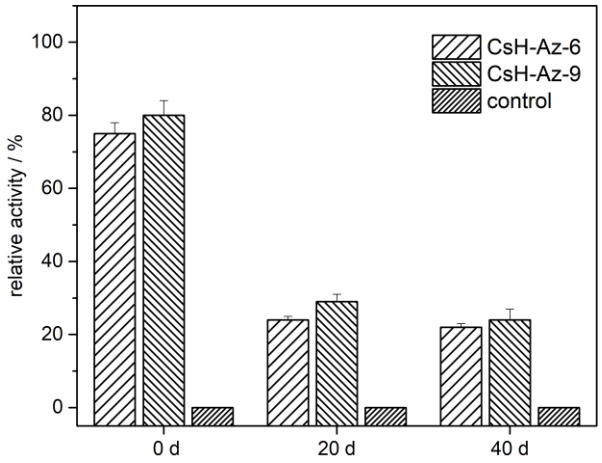

Figure 5: Remaining relative catalytic ability of β-Gal immobilized on photocrosslinked nanofiber samples after 0, 20 and 40 d of incubation in Millipore water.

Relative immobilized enzyme activity was reduced to under 30 % after storage of 20 to 40 days. These results suggest a good initial loading capacity of CsH-Az nanofibers but also a significant loss of activity over time. This could be attributed to leaching of enzymes or loss of active structure. Nevertheless, it can be concluded that at least 22 % of active enzymes were present on CsH-Az nanofibers after 40 days.

CONCLUSIONS

Adapting an electrospinning process for chitosan derivatives bearing photoreactive arylazide groups was accomplished using Methanol as co-solvent. Photoreactive arylazide groups from CsH-Az were used to induce crosslinking of polymer networks via irradiation with UV-light. Fully crosslinked fibers were shown to form a hydrogel-like structure when incubated in water or acidic buffer systems but retain fibrous morphology. Applicability as promising enzyme immobilization matrix was proven with β-*D*-galactosidase as model enzyme. After initial leaching during the first days, remaining catalytic abilities of immobilized enzymes could be retained over 40 days of incubation. These findings only hint the full potential of nanofibers from photoreactive chitosan, which is still subject of our ongoing research.

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REFERENCES

Ding, F., Deng, H., Du, Y., Shi, X., Wang, Q., 2014. Emerging chitin and chitosan nanofibrous materials for biomedical applications. *Nanoscale* 6 (16), 9477–9493.

Gan, Q., Wang, T., 2007. Chitosan nanoparticle as protein delivery carrier--systematic examination of fabrication conditions for efficient loading and release. *Colloids Surf., B* 59 (1), 24–34.

Hadler, C., Wissel, K., Brandes, G., Dempwolf, W., Reuter, G., Lenarz, T., Menzel, H., 2017. Photochemical coating of Kapton(R) with hydrophilic polymers for the improvement of neural implants. *Mater. Sci. Eng., C* 75, 286–296.

Li, Q., Wang, X., Lou, X., Yuan, H., Tu, H., Li, B., Zhang, Y., 2015. Genipin-crosslinked electrospun chitosan nanofibers: Determination of crosslinking conditions and evaluation of cytocompatibility. *Carbohydr. Polym.* 130, 166–174.

Misson, M., Zhang, H., Jin, B., 2015. Nanobiocatalyst advancements and bioprocessing applications. *J. R. Soc., Interface* 12 (102), 20140891.

Sarkar, S.D., Farrugia, B.L., Dargaville, T.R., Dhara, S., 2013. Physico-chemical/biological properties of tripolyphosphate cross-linked chitosan based nanofibers. *Mater. Sci. Eng.*, C 33 (3), 1446–1454.

Schiffman, J.D., Schauer, C.L., 2007. Cross-linking chitosan nanofibers. *Biomacromolecules* 8 (2), 594–601.

Sheldon, R.A., 2007. Enzyme Immobilization: The Quest for Optimum Performance. *Adv. Synth. Catal*. 349 (8-9), 1289–1307.

Zhang, Y.Z., Su, B., Ramakrishna, S., Lim, C.T., 2008. Chitosan nanofibers from an easily electrospinnable UHMWPEO-doped chitosan solution system. *Biomacromolecules* 9 (1), 136–141.

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